

# Particle Size Distributions for Cellulose Nanocrystals Measured by Transmission Electron Microscopy: An Interlaboratory Comparison

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**ABSTRACT:** Particle size is a key parameter that must be measured to ensure reproducible production of cellulose nanocrystals (CNCs) and to achieve reliable performance metrics for specific CNC applications. Nevertheless, size measurements for CNCs are challenging due to their broad size distribution, irregular rod-shaped particles, and propensity to aggregate and agglomerate. We report an interlaboratory comparison (ILC) that tests transmission electron microscopy (TEM) protocols for image acquisition and analysis. Samples of CNCs were prepared on TEM grids in a single laboratory, and detailed data acquisition and analysis protocols were provided to participants. CNCs were imaged and the size of individual particles was analyzed in ten participating laboratories that represent a cross section of academic, industrial, and government laboratories with varying levels of experience with imaging CNCs. The data for each laboratory were fit to a skew normal distribution that accommodates the variability in central location and distribution width and asymmetries for the various data sets. Consensus values were obtained by modelling the variation between laboratories using a skew normal distribution. This approach gave consensus distributions with values for mean, standard deviation and shape factor of 95.8 nm, 38.2 nm and 6.3 for length and 7.7 nm, 2.2 nm and 2.9 for width. Comparison of the degree of overlap between distributions for individual laboratories indicates that differences in imaging resolution contribute to the variation in measured widths. We conclude that the selection of individual CNCs for analysis and the variability in CNC agglomeration and staining are the main factors that lead to variations in measured length and width between laboratories.

Cellulose nanocrystals (CNCs) are emerging nanomaterials derived from cellulose, the world's most abundant biopolymer.<sup>1,2</sup> Their large-scale production from a renewable resource, novel properties, and anticipated minimal toxicity make them an attractive candidate for a range of applications, some of which are nearing commercial success.<sup>3-6</sup> Application areas include additives for nanocomposites, thin films, rheology modifiers, and biomedical products. CNCs are produced from a number of cellulosic biomass sources, including trees and annual plants, bacteria and marine organisms. They are nanorods with a typi-

cal aspect ratio of  $\approx 15$  (for wood pulp CNCs), with high crystalline cellulose content, high mechanical strength and a low coefficient of thermal expansion. Most CNC production methods generate negatively charged groups on the particle surface, which provide colloidally stable dispersions and sites for further functionalization. Development of applications for this novel nanomaterial requires reliable characterization protocols that can be used to ensure reproducible production of the material from various biomass sources and numerous producers. Validated characterization methods and reference materials are also

necessary for studies aimed at ensuring the safe use of these materials.<sup>7</sup>

Key properties that must be assessed for CNCs include size and shape, crystallinity, and surface functional groups. It is challenging to measure CNC particle size distributions due to their irregular rod-shaped structure, broad size distributions, and strong tendency to agglomerate and aggregate. This is in contrast to well-behaved, spherical nanoparticles with narrow monomodal size distributions. Both transmission electron microscopy (TEM) and atomic force microscopy (AFM) have been employed by the CNC community for particle size measurements, although a standard approach for sample preparation, imaging and image analysis is still lacking.<sup>8-13</sup> We report here the results of an interlaboratory comparison (ILC) conducted under the auspices of the Versailles Project on Advanced Materials and Standards (VAMAS) Technical Working Area 34 (Nanoparticle Populations). The objective was to assess the performance of protocols for TEM and AFM imaging of CNCs and subsequent particle size analysis. In order to reduce the impact of sample preparation on variability of specimens for imaging, the ILC used a National Research Council Canada (NRCC) reference material, CNCD-1, with samples prepared on substrates by the piloting laboratory. CNCD-1 was produced by sulfuric acid hydrolysis of softwood pulp, followed by neutralization with NaOH and spray drying and has been extensively characterized.<sup>14</sup> The results of this study will provide the prenormative data necessary for the development of a technical specification on measurement of CNC particle size distribution at ISO/TC 229 – Nanotechnologies. This paper focuses on a summary of the TEM results.

Transmission electron microscopy is generally considered the most reliable method for obtaining particle size distributions for nanomaterials. Most studies that have developed and validated TEM measurement protocols have employed gold, silica or polystyrene nanoparticle reference materials.<sup>15-17</sup> These studies have been supported through international ILCs that allow one to assess measurement uncertainty and comparability across multiple laboratories. Nevertheless, these studies have utilized approximately spherical nanoparticles with low polydispersity and good TEM contrast and are not immediately adaptable to more complex “real-life” nanomaterials such as those produced industrially. One recent study has begun to address this complexity by using a combination of particle counting, fractionation, and spectroscopy methods to evaluate the size distribution for a series of industrial nanomaterials with complex shapes and significant polydispersity; the results were compared to those obtained for well-defined spherical and monodisperse quality control samples.<sup>18</sup> It was concluded that a tiered approach using methods such as dynamic light scattering (DLS) for an initial screen and TEM for more detailed studies was appropriate for particle sizing. The issues associated with TEM measurements for nanomaterials with more complex shapes and strong agglomeration/aggregation have also recently been examined in a series of ILCs for gold nanorods, titanium dioxide nanoparticles, and carbon black.<sup>19-21</sup> These studies focused on a detailed assessment of image analysis methods for measurement of particle size distributions and provided a full uncertainty analysis, an essential feature in order to test for batch-to-batch similarity of materials or compare different CNC sources or preparation methods. They also provided the basis for publication of an ISO standard on TEM measurements of nanoparticles.<sup>22</sup>

Although the TEM protocols mentioned above provide a good basis for addressing some of the issues encountered for polydisperse and non-spherical nanomaterials, there are additional factors that require consideration for CNCs. Their shape is considerably less regular than that of the gold nanorods studied earlier; their TEM contrast, even after staining, makes it more difficult to distinguish CNCs from background; and the level of agglomeration is high, similar to the aggregates observed for TiO<sub>2</sub> or carbon black. These issues motivated this ILC, in which we focus on validating a measurement and analysis protocol that can be used to obtain CNC particle size distributions and assess uncertainty of the measurements. TEM particle size data from 10 participating laboratories were modelled using skew normal distributions for the dispersion of the measurement results provided by each laboratory as well as the dispersion that is observed between the laboratories.

## Experimental Section

**Sample preparation.** An NRCC certified reference material, CNCD-1,<sup>23</sup> was dispersed by ultrasonication to give a 2 % mass fraction CNC suspension that was deposited on plasma-treated carbon-coated copper grids and stained with uranyl acetate. A previously reported procedure<sup>14, 24</sup> was optimized as outlined in the Supporting Information. ILC samples were prepared from a single CNC suspension on three separate days over an 11-day period to ensure that each laboratory received the sample on a pre-arranged (convenient) date. One sample from each day was checked by TEM prior to shipping the samples and DLS was used to verify that the suspension had not changed. Samples were sealed in a nitrogen glove box prior to shipping. No change in image quality was observed over a 5-week storage period for samples of CNCs deposited on the TEM grid

**Participating laboratories.** The participating institutions were from Asia, Europe, and North America. These included three universities, six government laboratories, and one industrial laboratory. Not all laboratories had prior experience with imaging CNCs. Two samples of CNCs on carbon-coated copper grids were sent to each participant; the second sample was a backup. Participants were asked to image the samples within 2 weeks of receipt. Of the original 11 participants (randomly assigned a code, T#), 10 completed the ILC and returned data sets.

**Image acquisition and analysis protocol.** The protocol requested the following operating conditions: (1) an operating voltage between 80 kV and 300 kV; (2)  $\approx 30\,000$  magnification, such that the resolution is  $\leq 0.3$  nm/pixel (3) calibration and lens normalization at the magnification used for CNC imaging; (4) acquisition of larger scale images (e.g.,  $2\,\mu\text{m} \times 2\,\mu\text{m}$  or larger) in multiple grid locations to locate suitable areas for higher magnification images; (5) collection of a sufficient number of images from multiple areas of the grid to analyze a minimum of 500 individual particles. Image analysis using a custom ImageJ macro was recommended but participants had the option to use other software as long as the guidelines for particle selection were used. The ImageJ macro allows for automatic sequential opening of a set of images and manual measurement of length and width cross sections and saves the data and annotated images with the line profiles for analyzed CNCs. The criteria for selection of individual CNCs are provided in the Supporting Information.

**Data Analysis.** Origin Pro 2019 was used for descriptive statistics and statistical comparison of data sets using the non-parametric two-sample Kolmogorov-Smirnov (K-S) test (e.g.,

length for lab 1 and lab 2). More complex data analysis and visualization was performed in R (free software environment for statistical computing and graphics). Skew normal model fitting was performed using Markov Chain Monte Carlo methods using STAN (a probabilistic programming language for statistical inference written in C++). The skew normal distribution differs from normal and lognormal distributions by the presence of an additional shape factor that allows for variable levels of skewness. Fitting was done using the individual data points, not the binned data shown in histograms. The overlap index used to compare particle size distributions was calculated by integrating the areas under curves using the standard numerical facilities of R.

## Results and Discussion

**Tests of Image Analysis Protocols.** The first phase of the ILC focused on testing ImageJ analysis methods. Three possible methods were considered. The first method was based on prior work in the piloting laboratory that used a semi-automated ImageJ macro to measure length and width manually for each particle.<sup>14, 24</sup> Two other methods that were employed in recent TEM ILCs that analyzed gold nanorods and (aggregated) titanium dioxide were evaluated.<sup>20, 21</sup> The contrast of stained CNCs against the substrate background was inadequate to utilize the automated approach based on thresholding that was employed for gold nanoparticles.<sup>20</sup> The method from the titanium dioxide ILC used the manual polygon outlining tool in ImageJ.<sup>21</sup> This manual outlining approach provides particle length and width (as maximum and minimum Feret, respectively) and other potentially useful parameters such as area and perimeter. Use of the polygon outlining tool gave length values in good agreement with the pilot laboratory's semi-automated ImageJ macro. However, the outlining tool consistently over-estimated width compared with manual measurement using the semi-automated ImageJ macro. We hypothesize that the irregular tapered shape of the CNCs is incompatible with the minimum Feret measurement of the minimum distance between the two parallel tangents touching the particle outline. Therefore, the manual method using the semi-automated ImageJ macro to keep track of measurement data was adopted and further optimized for this ILC.

A set of images was provided to ILC participants to test the image analysis protocol. The images were a subset of those collected for one sample as part of the characterization of the NRCC certified reference material, CNCD-1.<sup>14</sup> Six data sets were analyzed: the original CNCD-1 data for these images plus data from four laboratories, one of which submitted data from two analysts. Comparison of the results indicated that participants interpreted the image analysis protocol instructions differently. This was evident from the number of CNCs analyzed which varied from 128 to 440. In one case, Olympus automated software was used for automated analysis (Lab 4). The box plots summarizing each data set are shown in Figure S1 and demonstrate significant variability in the data. The means for length and width ranged from 71 nm to 105 nm and 7.0 nm to 10.0 nm, respectively (Table S1). K-S analysis indicated that all but two of the 15 possible pairs of data sets were significantly different for both length and width; the labs with no significant difference were different for the two measurands (Table S1).

Based on these observations and the feedback from participants, the image analysis protocol was modified to provide more detailed instructions for the selection of analyzable CNCs. Images

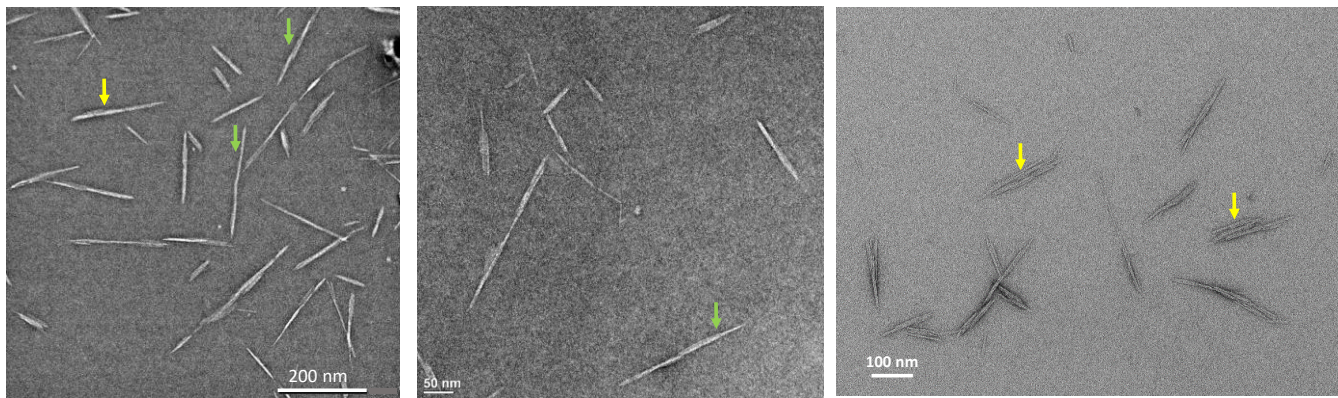
with examples of analyzable CNCs and CNCs that should be excluded from size analysis were also provided as an Annex to the protocol (see Figures S2, S3 and S4). The improved protocol was used for CNC size analysis in the second phase of the ILC.

**Initial Survey of TEM Results.** Results were submitted from 10 participating laboratories; each participant submitted raw data (images), processed images with analyzed particles numbered, tables of length and width for each particle analyzed, length and width histograms and a table with data acquisition and analysis information. The instruments, operating parameters, calibration, analysis software and number of particles analyzed for each laboratory are summarized in Table S2. The resolution varied from  $\approx 0.2$  nm/pixel to 0.46 nm/pixel, with most labs collecting data at a resolution that was  $\leq 20$  % larger than the recommended value of  $\leq 0.3$  nm/pixel. Lab 1 collected images using two magnifications; a K-S test indicated that there was no significant difference between the two data sets, so they were pooled for further analysis. In several cases, participants were asked to verify that the reported calibration was still valid. Most participants (9 of 10) reported data for 500 or more analyzed CNCs as requested; the largest data set had 1179 particles and the smallest had 323 particles. The relatively small number of individual CNCs/image (typically 5 to 12 CNCs per  $\mu\text{m}^2$ ) meant that all labs collected more than 50 images, making it impractical to compare intra-laboratory repeatability on an image-by-image basis.

Representative images from three laboratories are shown in Figure 1. These images illustrate the variations in contrast and the significant agglomeration of CNCs that is typically observed, even at low CNC density. See, for example, the arrows that highlight small clusters of laterally agglomerated CNCs in the T9 image. The number of features assigned to clustered CNCs is typically similar to or larger than the number of individual particles. The images from T3 and T6 have several irregularly shaped features that appear to be end-to-end CNCs (green arrows) and laterally agglomerated CNCs (yellow arrow). Note that such features may be counted in some cases but not others, depending on the image contrast and resolution and the analyst bias. Box plots of the length and width data for each laboratory are shown in Figure S5, and individual histograms for length, width and aspect ratio for each laboratory are provided in Figures S6, S7 and S8, respectively. In several cases, examination of the box plots showed a few data points that suggested errors in reported data; this was verified by examining the image files and consulting the participant as to whether the data point(s) should be removed or corrected.

The results in Figure S5 to S8 show qualitatively that the maximum position, shape, and width of the distributions vary from one data set to the next for both length and width. The variability between laboratories is somewhat lower for aspect ratio, as compared to length and width. Operator bias when deciding whether irregular features (such as those noted in Figure 1) are single CNCs contribute to this variability

**Particle size distributions.** Statistical modeling of interlaboratory results requires a selection of adequate probability density functions that describe the data at hand. Microscopy size measurements for CNCs typically provide data in the form of histograms and calculate the mean and the standard deviation as a measure of the distribution width, with the implicit assumption that the distributions fit a normal model. In several cases the data have been shown to provide reasonable fits to a lognormal distribution.<sup>25-28</sup> Recent ILC studies of other nanomaterials have



**Figure 1.** Representative TEM images from labs T3 (left), T6 (centre) and T9 (right). The arrows mark features that are discussed in the text.

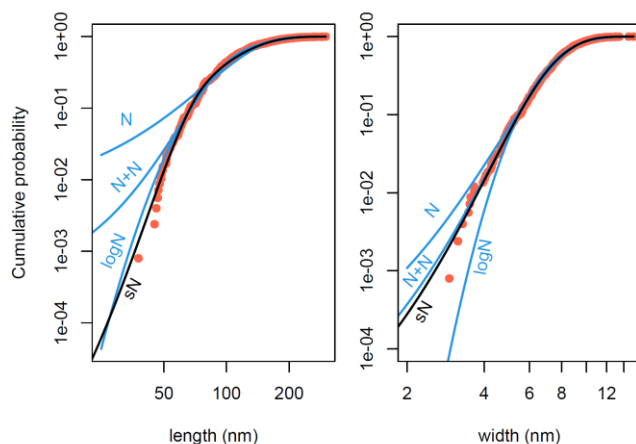
tested various distribution models in an attempt to better represent the overall central location and breadth/shape of the distributions. For example, the titanium dioxide ILC compared normal, lognormal, and Weibull distributions<sup>21</sup> whereas a recent comparison of electron microscopy and ICP-MS data for gold nanoparticles employed mixtures of Gaussian distributions to model the complex shapes of the particle size distributions.<sup>29</sup> Others have adopted different statistical models for this purpose, for example by employing a (discrete) multinomial distribution.<sup>30</sup> Model selection has so far been largely empirical; furthermore, the CNC extraction method differs from bottom-up synthetic methods for metal or metal oxide nanoparticles so there is no reason to anticipate that the same size distribution model will apply.

We have evaluated the particle size distributions against more than 50 continuous probability distributions using the tools implemented in the R package GAMLSS.<sup>31</sup> Probability density functions were fitted to data using the maximum likelihood method and ranked by the quality of each model as measured by the Bayesian Information Criterion (*BIC*). Several distributions produced a reasonable fit for our data sets, the simplest of which are the lognormal and skew normal distributions. Figure 2 shows the comparison of four statistical models applied to the same data sets. These models are of varied complexity ranging from 2-parameters (normal or lognormal distributions), to 3-parameters (skew normal), to 5-parameters (two-mixture normal).

While one can always find a complex distribution that best fits a particular data set, the results indicate that the skew normal distribution strikes a balance of being a model that is simple (three parameters) and flexible (allows positive and negative skew), and fits the data very well. The skew normal distribution reduces to the normal distribution for data sets that show no asymmetry. Therefore, it is applicable to data sets that fit either normal or lognormal distributions; the use of a single distribution facilitates comparison between data sets from different laboratories and the development of a consensus distribution, as explained in the following section.

The skew normal distribution (sN) is relatively new<sup>32</sup> and has been used to model grain-size distributions<sup>33</sup> among other applications.<sup>34</sup> Its probability density is derived by multiplying the normal distribution by the standard error function. The skew normal distribution has 3 parameters: location ( $\mu$ ), scale ( $\sigma$ , a measure of distribution width) and shape ( $\alpha$ , a measure of dis-

tribution skew or asymmetry). Note that several parametrizations have been introduced to improve its mathematical performance (such as numerical stability or sampling efficiency).



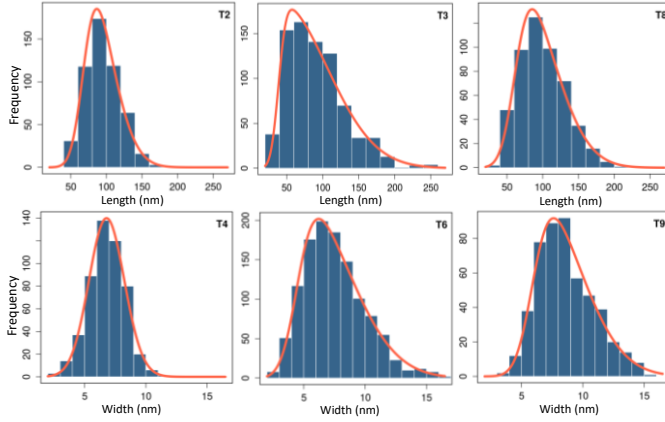
**Figure 2.** Log-log cumulative probability plots for CNC length and width for laboratory T1 with fits of normal (N), lognormal (logN), skew normal (sN), and 2-mixture normal (N+N) distributions. The sN distribution provides a better fit than the other three models for both data sets.

The fitting was performed with the Markov Chain Monte Carlo method using STAN (a probabilistic programming language for statistical inference) and R. The facilities to construct and fit skew normal Bayesian models have been developed in the R package brms.<sup>35</sup> As an example, the following general syntax can be utilized to perform Bayesian fitting of the skew normal distribution:

$$\text{brm}(\text{length} \sim 1, \text{data}, \text{family}=\text{skew\_normal}) \quad \text{eq. 1}$$

Fits to a skew normal distribution for TEM length and width are shown for several representative data sets in Figure 3; all data sets are shown in Figures S6-S8. Table 1 summarizes mean, scale (as standard deviation), and skew normal shape parameters for length, width, and aspect ratio for each laboratory. Overall, the length distributions are broader (based on the relative standard deviations of their skew normal distributions) than the width distributions and show larger variability between laboratories. The fitted skew normal distribution shape factors show a

rough correlation with the scale/mean ratio for both length and width.



**Figure 3.** Representative data sets showing TEM histograms for length (top row) and width (bottom row) and a skew normal fit for the particle size distribution. Data sets were selected to show the range of distribution widths and shapes.

**Consensus distribution, parameters and uncertainty.** Data reduction from interlaboratory studies can be conducted in several ways. An approach that makes the fewest modeling assumptions is to pool all the results together. Alternatively, one can establish a statistical model for individual laboratory results and the dispersion between the laboratories. The latter is formally known as the hierarchical random effects model. The general principles of applying a random effects model to interlaboratory studies have been outlined by Toman and Possolo<sup>36</sup> and Koepke *et al.*<sup>37</sup> More recently, similar models have been extended to deal with data exhibiting asymmetric tendencies.<sup>38</sup>

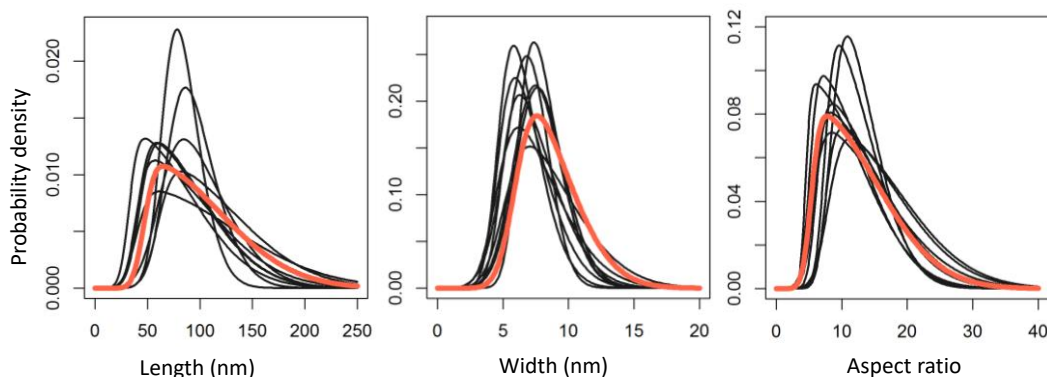
The statistical model of our ILC study follows the general principles of Montoro Bustos *et al.*<sup>29</sup> (pooling all results) with the exception of using skew normal distribution to model the particle size distribution, rather than mixtures of Gaussian distributions. The skew normal consensus distribution was obtained by pooling the skew normal distributions representing the individual laboratory results. An equal number of random samples ( $N = 1000$ ) was drawn from each of the skew normal distributions and placed into the pool. The statistical model of the entire ILC study (for each measurand) is therefore summarized by 3 parameters for each laboratory along with 3 additional parameters describing the distribution of consensus values, the grand mean, scale, and shape.

The TEM data contain a total of 6168 observations from 10 laboratories for each of the measurands (width, length, and aspect ratio) and the fitting results for each laboratory and the consensus distribution are summarized in Figure 4. The final consensus data for length, width, and aspect ratio and the associated uncertainties are summarized in Table 1. The uncertainties are obtained as a direct output of fitting to the skew normal model. The dispersion between the mean laboratory results ( $t$ ) is also provided as a measure of variability between the laboratories. The  $t$  values are all substantially larger than their uncertainties, which is indicative of significant spread in the data sets as illustrated qualitatively by the histograms, box plots and fitted parameters in Table 1. The dispersion value allows predictive assessment of how close the individual laboratory means should be to the consensus value.

**Table 1.** Summary of skew normal fit parameters for individual lab data and the consensus distribution for length, width, and aspect ratio measurements by TEM.

Laboratory	Length			Width			Aspect ratio		
	Mean (nm)	Std. dev. (nm)	sN shape, $\alpha$	Mean (nm)	Std. dev. (nm)	sN shape, $\alpha$	Mean	Std. dev.	sN shape, $\alpha$
T1	116.6	44.5	6.6	7.8	1.9	1.5	15.7	6.4	9.5
T2	94.5	23.9	2.5	8.0	1.9	1.6	12.6	4.1	5.9
T3	91.9	41.1	8.0	7.5	2.1	4.0	12.9	5.4	6.9
T4	83.8	18.4	2.2	6.8	1.6	0.0	12.9	3.8	3.7
T5	86.4	35.8	6.7	8.0	1.6	2.8	11.1	4.8	8.2
T6	77.8	35.2	8.4	7.5	2.5	3.7	11.1	5.0	13.2
T7	87.6	35.6	6.1	6.9	1.9	3.5	13.5	5.8	9.7
T8	99.9	33.0	3.2	6.5	1.7	2.9	16.3	6.6	6.4
T9	102.8	43.3	8.8	8.7	2.3	3.2	13.0	5.9	9.0
T11	110.2	54.7	9.3	8.6	2.9	3.7	13.8	6.5	7.6
Consensus value (standard uncertainty)	95.8 (0.4)	39.0 (0.3)	4.8 (0.2)	7.65 (0.02)	2.20 (0.02)	2.8 (0.1)	13.3 (0.06)	5.69 (0.05)	6.4 (0.3)
Dispersion, $t$ (standard uncertainty)	12.3 (0.5)			0.78 (0.03)			1.8 (0.1)		



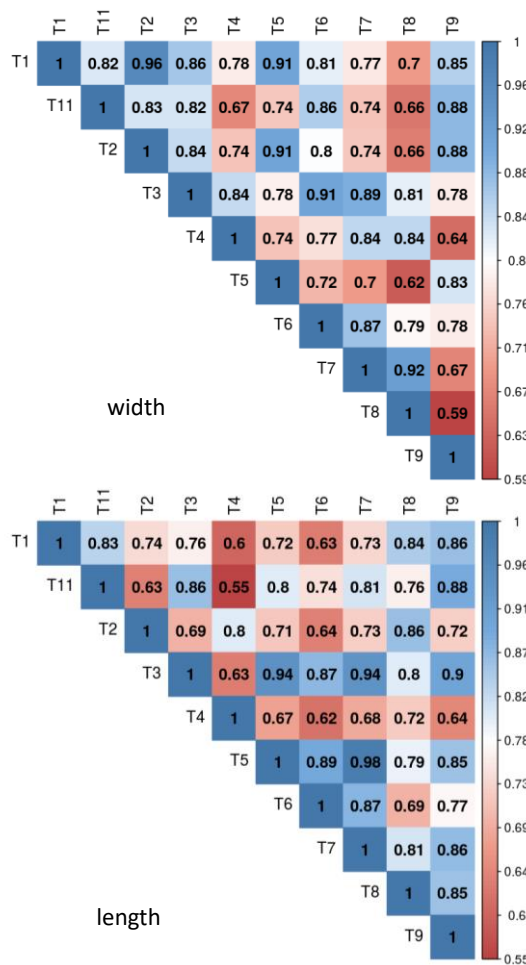


**Figure 4.** Skew normal probability densities describing the individual laboratory results (black lines) and the corresponding consensus distribution (orange line).

**Data comparisons.** The variability in skew normal distributions for the individual laboratories was examined using an overlap index which is defined as the relative common area between two distributions.<sup>39</sup> This is shown graphically in Figure 5, which provides the degree of overlap between individual data sets for both length and width. The mean, scale and shape parameters and degree of overlap (Figure 5) can be used to test for correlations with changes in the experimental parameters used by the participating laboratories (Table S2). For width, three data sets have means that are smaller than 7 nm (T4, T7 and T8, Table 1) and all are measured at the best microscope resolution (0.20, 0.22, and 0.25 nm/pixel, Table S2). These three data sets have narrower particle width distributions as measured by the scale parameter and two (T4 and T8) have the lowest average overlap (estimated as the average of the overlap with all other data sets, Figure 5) with other data sets. Overall, the results suggest that a resolution of 0.2 nm/pixel would give a more accurate estimate of width. However, there is a trade-off since that would generally require collection and analysis of a larger number of images to obtain the desired number of single particles for size analysis. By contrast, the data set measured at the lowest resolution (T2, 0.46 nm/pixel) has a mean and scale that are close to the consensus values, which indicates that factors other than the resolution contribute to differences between laboratories. There is no apparent correlation with the number of CNCs analyzed, as the data sets with the highest (T6) and lowest (T5) N values have mean width and scale values that are close to the overall consensus values (and used similar imaging resolutions) and an intermediate degree of overlap with other data sets. For pairwise comparisons (Figure 5), T8 and T9 have the lowest overlap of all pairs (0.59) followed by T8 and T5 (0.62). The low overlap reflects relatively large differences in both scale and mean for these two data sets.

The length overlap index graphic in Figure 5 indicates that the pairs of labs with the lowest overlap are T11/T4 (0.55) and T4/T1 (0.6); these variations are due to large differences in mean length and for the T4/T1 pair the largest difference in distribution width (scales of  $\approx 18$  nm and  $\approx 55$  nm for T4 and T1). The two data sets which show the lowest degree of overlap with other laboratories (T4 and T2) have the smallest scale factors (18 nm and 24 nm), similar to the observations for width above; the small scale factor is mainly due to the absence of longer CNCs, as illustrated in Figure 1. These data sets were measured using different resolutions (0.46 nm/pixel and 0.22 nm/pixel, Table S2), consistent with the expectation that particle length is insensitive to changes in resolution. Interestingly these two labs

have mean lengths that are close to the overall consensus value. The degree of overlap for length is lowest for the T4/T11 pair (0.55) and highest for the T5/T7 pair (0.98) which have almost the same mean and scale parameters. The data sets with the largest difference in number of CNCs analyzed (323 and 1179 CNCs for T5 and T6, respectively) have similar scales and



**Figure 5.** Degree of overlap (overlap index; relative common area between the two distributions) between data sets for width and length distributions.

smaller means than the consensus value, providing little evidence that the number of CNCs analyzed has a major effect over the range covered here.

To test whether data sets with < 500 analyzed CNCs yield repeatable results for a specific laboratory, smaller data subsets were compared for the two laboratories with the largest number of analyzed CNCs (T3 and T6). K-S tests showed that particle width distributions for data sets of  $\approx 280$  CNCs were different for both labs. By contrast, the length distributions for the data subsets were different for T3, but not for T6. The number of points needed to obtain stable size measurements was also examined by plotting the cumulative mean as a function of the number of analyzed particles as shown in Figure S9 for several data sets. This shows that the mean length stabilizes with 200–300 analyzed particles in some cases but not others. The TEM width variations are similar to a one pixel error for some data sets (pixel sizes of 0.2 to 0.4 nm for the labs shown); however, in other cases the width continues to vary with an increasing number of analyzed particles. Although more detailed studies are required to make a definitive conclusion, we hypothesize that variations in CNC agglomeration and staining for different grid areas may account for these results. This issue makes it difficult to establish an optimal number of analyzed particles without verifying whether a stable particle length and width are obtained. Comparison of length and width skew normal fit parameters indicates that the magnitude of the mean and scale parameters follow a similar pattern for individual laboratories. Data set T11 has the largest scale factor and the second largest mean for both length and width. This does not appear to correlate with the resolution used or the number of CNCs analyzed. Although the T11 data set was obtained using different analysis software, visual inspection of analyzed images provides no indication that the selection of analyzable CNCs is different for this laboratory. The mean and scale for data set T9 are also among the largest for both length and width and T4 has the smallest scale and second smallest mean for length and the second smallest mean and scale for width. Based on the initial image analysis tests, it can be concluded that the selection of individual CNCs for analysis is a significant contributor to differences between laboratories.

The consensus values can be compared to the data reported for CNCD-1, which was characterized by TEM in two laboratories. The reported values for mean (standard deviation) were 87 (35) nm and 7.3 (1.8) for length and width, respectively, for the combined data from the two laboratories.<sup>14</sup> These values fall within the range of results obtained from the ten ILC laboratories (Table 1); however, the ILC data indicate that a mean value of  $\approx 96$  nm provides a better estimate of the CNC length for CNCD-1 than the previously reported value of 87 nm.<sup>23</sup> It should also be noted that the original data was not obtained by fitting to a skew normal distribution which is anticipated to lead to a small (1 nm to 2 nm) difference in values. The previous study examined 5 independently prepared samples, all of which were analyzed by a single analyst;<sup>14</sup> the variability in length was lower than observed in the ILC, although the width variability was similar. The latter indicates that variations in length for the ILC include contributions from sample-to-sample variations and differences between laboratories/analysts.

As noted above, analyst bias/subjectivity and sample heterogeneity are the main sources of ILC variability. The subjectivity in choice of analyzable CNCs can in principle be reduced by use of automated image analysis methods that are currently be-

ing developed. Although significant effort was made to optimize the CNC deposition and staining method used to prepare samples for this ILC, agglomeration and uneven staining remain an issue. Recent work has demonstrated that fractionation of CNCs using asymmetric flow field flow fractionation can significantly reduce the level of CNC agglomerates, yielding samples that almost eliminate clusters as assessed by AFM imaging.<sup>40</sup> The fractionated samples show some improvement in dispersion on TEM grids, although there are significantly more clusters than observed in samples deposited for AFM.

## Conclusions

The ILC data obtained from ten laboratories show variability in the central location, width and asymmetry of the measured particle size distributions for length and width. In order to make quantitative comparisons between individual data sets and facilitate development of consensus values we have employed an approach wherein each data set is fit to a skew normal distribution which yields three parameters: the mean as a measure of the central location, standard deviation as a measure of the distribution scale/width and a shape parameter as a measure of the distribution asymmetry. This approach has the advantage of using a single distribution that can adequately deal with the variability in the data while introducing only one new fit parameter compared to the two-parameter distributions, such as normal or lognormal, that have frequently been employed for fitting particle size distributions. This approach also has fewer parameters than fits to sums of distributions.

Consensus values are obtained from the individual data sets by modelling the variation between laboratories using a skew normal distribution. This gives values for the consensus distribution for mean, standard deviation and shape of 95.8 nm, 38.2 nm and 6.3, respectively, for length and 7.7 nm, 2.2 nm and 2.9, respectively, for width, with uncertainties as listed in Table 1. Attempts to correlate the lab-to-lab variability with differences between experimental conditions for data acquisition and analysis indicated that a higher magnification for image acquisition yielded particle width distributions with smaller mean and standard deviation values. One can conclude that a resolution of < 0.3 nm/pixel would be preferable for CNC imaging, although collection of additional images may be necessary to obtain the required number of particles for analysis and may lead to rejection of a larger fraction of long particles. However, the use of a smaller pixel size did not appear to be a factor in determining the measured differences in length. Overall, we conclude that the selection of individual particles for image analysis is one of the larger contributors to differences between laboratories, even after implementing an improved analysis protocol based on the results of the image analysis tests in phase 1. Variability in CNC agglomeration/aggregation and staining<sup>11</sup> is another important factor; this is difficult to control even after optimizing the protocol and preparing all samples in the piloting laboratory.

Finally, we note that the AFM imaging results obtained during this ILC will be reported elsewhere. A final comparison of size measurements from the two microscopy methods will provide a useful assessment of methods for image acquisition and analysis procedures for CNCs. It will also give insight on the agreement between laboratories and the approaches necessary to make comparisons between different CNC sources and their characterization in different laboratories.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Additional experimental details and tables and figures summarizing the image analysis protocol and tests and histograms and fits for all data sets

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#### Notes

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**DISCLAIMER:** Any mention of commercial products is for information only; it does not imply recommendation or endorsement by NIST.

## REFERENCES

1. Moon, R.J.; Martini, A.; Nairn, J.; Simonsen, J.; Youngblood, J. Cellulose nanomaterials review: Structure, properties and nanocomposites. *Chem. Soc. Rev.* **2011**, *40*, 3941-3994.
2. Klemm, D.; Kramer, F.; Moritz, S.; Lindstrom, T.; Ankerfors, M.; Gray, D.; Dorris, A. Nanocelluloses: A new family of nature-based materials. *Angew. Chem. Int. Ed. Engl.* **2011**, *50*, 5438-5466.
3. Shatkin, J.A.; Wegner, T.H.; Bilek, E.M.; Cowie, J. Market projections of cellulose nanomaterial-enabled products- part 1: Applications. *TAPPI J.* **2014**, *13*, 9-16.
4. Grishkewich, N.; Mohammed, N.; Tang, J.; Tam, C. Recent advances in the application of cellulose nanocrystals. *Curr. Opin. Colloid Inter. Sci.* **2017**, *29*, 32-45.
5. Mondal, S. Preparation, properties and applications of nanocellulosic materials. *Carbohydr. Poly.* **2017**, *163*, 301-316.
6. Mokheba, T.C.; John, M.J. Cellulose nanomaterials: New generation materials for solving global issues. *Cellulose* **2020**, *27*, 1149-1194.
7. Shatkin, J.A.; Kim, B. Cellulose nanomaterials: Life cycle risk assessment and environmental health and safety roadmap. *Environ. Sci.: Nano* **2015**, *2*, 477-499.
8. Foster, E.J.; Moon, R.J.; Agarwal, U.P.; Bortner, M.J.; Bras, J.; Camarero-Espinosa, S.; Chen, K.J.; Clift, M.J.D.; Cranston, E.D.; Eichhorn, S.J.; Fox, D.M.; Hamad, W.Y.; Heux, L.; Jean, B.; Korey, M.; Nieh, W.; Ong, K.J.; Reid, M.S.; Renneckar, S.; Roberts, R.; Shatkin, J.A.; Simonsen, J.; Stinson-Bagby, K.; Wanasekara, N.; Youngblood, J. Current characterization methods for cellulose nanomaterials. *Chem. Soc. Rev.* **2018**, *47*, 2609-2679.
9. Kaushik, M.; Fraschini, C.; Chauve, G.; Putaux, J.-L.; Moores, A., Transmission electron microscopy for the characterization of cellulose nanocrystals, in *The transmission electron microscope - theory and applications*, K. Maaz, Editor. 2015, Intech. p. 129-163.
10. Ogawa, Y.; Putaux, J.-L. Transmission electron microscopy of cellulose. Part 2: Technical and practical aspects. *Cellulose* **2019**, *26*, 17-34.
11. Stinson-Bagby, K.L.; Roberts, R.; Foster, E.J. Effective cellulose nanocrystal imaging using transmission electron microscopy. *Carbohydr. Poly.* **2018**, *186*, 429-438.
12. Moon, R.J.; Pohler, T.; Tammelin, T., Microscopic characterization of nanofibers and nanocrystals, in *Handbook of green materials*, K. Oksman, et al., Editors. 2014, World Scientific: Singapore. p. 159-180.
13. Sacui, I.A.; Nieuwendaal, R.C.; Burnett, D.J.; Stranick, S.J.; Jorfi, M.; Weder, C.; Foster, J.E.; Olsson, R.T.; Gilman, J.W. Comparison of the properties of cellulose nanocrystals and cellulose nanofibrils isolated from bacteria, tunicate and wood processed using acid, enzymatic, mechanical and oxidative methods. *ACS Appl. Mat. Inter.* **2014**, *6*, 6127-6138.
14. Jakubek, Z.J.; Chen, M.; Couillard, M.; Leng, T.; Liu, L.; Zou, S.; Baxa, U.; Clogston, J.D.; Hamad, W.; Johnston, L.J. Characterization challenges for a cellulose nanocrystal reference material: Dispersion and particle size distributions *J. Nanopart. Res.* **2018**, *20*, 98.
15. Rice, S.B.; Chan, C.; Brown, S.C.; Eschbach, P.; Han, L.; Esnor, D.S.; Stefaniak, A.B.; Bonevich, J.; Vladar, A.E.; Hight Walker, A.R.; Zheng, J.; Starnes, C.; Stromberg, A.; Ye, J.; Grulke, E.A. Particle size distributions by transmission electron microscopy: An interlaboratory comparison case study. *Metrologia* **2013**, *50*, 663-678.
16. De Temmerman, P.-J.; Lammertyn, J.; De Ketelare, B.; Kestens, V.; Roebben, G.; Verleysen, E.; Mast, J. Measurement uncertainties of size, shape, and surface measurements using transmission electron microscopy of near-monodisperse, near-spherical nanoparticles. *J. Nanopart. Res.* **2014**, *16*, 2177.
17. Meli, F.; Klein, T.; Buhr, E.; Frase, C.G.; Gleber, G.; Krumrey, M.; Duta, A.; Duta, S.; Korpelainen, V.; Bellotti, R.; Picotto, G.B.; Boyd, R.D.; Cuenat, A. Traceable size determination of nanoparticles, a comparison among European metrology institutes. *Meas. Sci. Tech.* **2012**, *23*, 125005.
18. Babick, F.; Mielke, J.; Wohlleben, W.; Weigel, S.; Hodoroaba, V.-D. How reliably can a material be classified as a nanomaterial? Available particle sizing techniques at work. *J. Nanopart. Res.* **2016**, *18*, 158.
19. Grulke, E.; B. Rice, S.; Xiong, J.; Yamamoto, K.; Yoon, T.H.; Thomson, K.; Saffaripour, M.; Smallwood, G.; Lambert, J.; Stromberg, A.; Macy, R.; Briot, N.; Qian, D. Size and shape distributions of carbon black aggregates by transmission electron microscopy. *Carbon* **2018**, *130*, 822-833.
20. Grulke, E.A.; Wu, X.; Ji, Y.; Buhr, E.; Yamamoto, K.; Song, N.W.; Stefaniak, A.B.; Schwegler-Berry, D.; Burchett, W.W.; Lambert, J.; Stromberg, A.J. Differentiating gold nanorod samples using particle size and shape distributions from transmission electron microscope images. *Metrologia* **2018**, *55*, 254-267.
21. Grulke, E.A.; Yamamoto, K.; Kumagi, K.; Hausler, I.; Osterle, W.; Ortel, E.; Hodoroaba, V.-D.; Brown, S.C.; Chan, C.; Zheng, J.; Yamamoto, K.; Yashiki, K.; Song, N.W.; Kim, Y.H.; Stefaniak, A.B.; Schwegler-Berry, D.; Coleman, V.A.; Jamting, A.K.; Hermann, J.; Arakawa, T.; Burchett, W.W.; Lambert, J.W.; Stromberg, A.J. Size and shape distributions of primary crystallites in titania aggregates. *Adv. Powder Tech.* **2017**, *28*, 1647-1659.
22. ISO 21363:2020 Nanotechnologies - measurements of particle size and shape distributions by transmission electron microscopy
23. <http://www.nrc.ca/crm>.
24. Brinkmann, A.; Chen, M.; Couillard, M.; Jakubek, Z.J.; Leng, T.; Johnston, L.J. Correlating cellulose nanocrystal particle size and surface area *Langmuir* **2016**, *32*, 6105-6114.
25. Elazzouzi-Hafraoui, S.; Nishiyama, Y.; Putaux, J.-L.; Heux, L.; Dubreuil, F.; Rochas, C. The shape and size distribution of crystalline nanoparticles prepared by acid hydrolysis of native cellulose. *Biomacromolecules* **2008**, *9*, 57-65.
26. Postek, M.T.; Vladar, A.; Dagata, J.; Farkas, N.; Ming, B.; Wagner, R.; Raman, A.; Moon, R.J.; Sabo, R.; Wegner, T.H.; Beecher,



- J. Development of the metrology and imaging of cellulose nanocrystals. *Meas. Sci. Tech.* **2011**, *22*, 024005.
27. Usov, I.; Nystrom, G.; Adamcik, J.; Handschin, S.; Schutz, C.; Fall, A.; Bergstrom, L.; Mezzenga, R. Understanding nanocellulose chirality and structure-properties relationship at the single fibril level. *Nature Comm.* **2015**, *6*, 7564.
  28. Meng, Y.; Wu, Q.; Young, T.M.; Huang, B.; Wang, S.; Li, Y. Analyzing three-dimensional structure and geometrical shape of individual cellulose nanocrystal from switchgrass. *Polymer Composites* **2017**, 2368-2377.
  29. Montoro Bustos, A.R.; Petersen, E.J.; Possolo, A.; Winchester, M.R. Post hoc interlaboratory comparison of single particle ICP-MS size measurements of NIST gold nanoparticle reference materials. *Anal. Chem.* **2015**, *87*, 8809-8817.
  30. Petersen, E.J.; Montoro Bustos, A.R.; Toman, B.; Johnson, M.E.; Ellefson, M.; Caceres, G.C.; Neuer, A.L.; Chan, Q.; Kemling, J.W.; Mader, B.; Murphy, K.; Roesslein, M. Determining what really counts: Modeling and measuring nanoparticle number concentrations. *Environ. Sci.: Nano* **2019**, *6*, 2876-2896.
  31. Voncken, L.; Albers, C.J.; Timmerman, M.E. Model selection in continuous test norming with gamlss. *Assessment* **2019**, *26*, 1329-1346.
  32. O'Hagan, A.; Leonard, T. Bayes estimation subject to uncertainty about parameter constraints. *Biometrika* **1976**, *63*, 201-203.
  33. Yuan, R.; Zhang, C.; Wang, X.; Zhu, R. Utilizing skew normal distribution to unmix grain-size distribution of swampy lakeshore: Example from Lake Ulungur, China. *Arab J. Geosci.* **2018**, *11*, 695.
  34. Lin, T.I.; Lee, J.C. Estimation and prediction in linear mixed models with skew-normal random effects for longitudinal data. *Statist. Med.* **2008**, *27*, 1490-1507.
  35. Bürkner, P.-C. Brms: An R package for Bayesian multilevel models using stan. *J. Statist. Software* **2017**, *80*, No. 1.
  36. Toman, B.; Possolo, A. Laboratory effects models for interlaboratory comparisons. *Accred. Qual. Assur.* **2009**, *14*, 553-563.
  37. Koepke, A.; Lafarge, T.; Possolo, A.; Toman, B. Consensus building for interlaboratory studies, key comparisons, and meta-analysis. *Metrologia* **2017**, *54*, S34-S62.
  38. Possolo, A.; Merkatas, C.; Bodnar, O. Asymmetrical uncertainties. *Metrologia* **2019**, *56*, 045009.
  39. Pastore, M.; Calcagni, A. Measuring distribution similarities between samples: A distribution-free overlapping index. *Front. Psychol.* **2019**, *10*, 1089.
  40. Chen, M.; Parot, J.; Mukherjee, A.; Couillard, M.; Zou, S.; Hackley, V.A.; Johnston, L.J. Characterization of size and aggregation for cellulose nanocrystal dispersions separated by asymmetrical-flow field-flow fractionation. *Cellulose* **2020**, *27*, 2015-2028.

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## Table of Contents (TOC) Graphic

